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INHIBITION OF *VERTICILLIUM SUCHLASPORIUM* AND OTHER NEMATOPHAGOUS FUNGI BY BACTERIA COLONISING *HETERODERA AVENAE* FEMALES

by

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Summary. *Pseudomonas fluorescens* and a bacterium with similar characteristics (isolate A) were obtained from females of *Heterodera avenae* which caused growth-inhibition 'halos' in colonies of *Verticillium suchlasporium* or *Acremonium* sp. from naturally infected young females of *H. avenae*. These bacteria partially inhibited the growth of the egg-parasites *Paecilomyces carneus* and *Acremonium* sp. on agar. Isolate A also inhibited the growth of *V. suchlasporium* while *Cladosporium cucumerinum*, a fungal plant pathogen, was unaffected indicating a certain degree of specificity in the mycostasis. The percentage of nematodes infected by nematophagous fungi belonging to the genus *Verticillium* was less in young females than for newly formed cysts. The young females may have been initially protected by mycostasis which gradually decreased allowing the *Verticillium* spp. to infect the newly-formed cysts. The importance of mycostasis in the understanding of the process by which *H. avenae* is infected by nematophagous fungi under field conditions has still to be investigated.

Nematophagous fungi colonise the roots of the plants (Kerry *et al.*, 1984) where they attack female and cyst nematodes killing their eggs (Kerry, 1981). These egg-parasites especially *Verticillium* spp. are good candidates to be developed for the biocontrol of cyst-nematodes (Kerry, 1986).

However, in order to use these fungi effectively as biocontrol agents it is necessary to study their physiology, especially in relation to the factors that may affect their survival in the rhizosphere and soil, where their hosts (female nematodes and cysts) live. Fungi in soils are subjected to antagonism by different organisms, but especially other fungi and bacteria. This phenomenon has been referred to as soil mycostasis (Jackson, 1957; Ko and Lockwood, 1970). Mankau (1962) found for the nematode-trapping fungus *Arthrobotrys arthrobotryoides*, reductions in the germination of conidia up to 90% in citrus soil. He also found that conidia of nematode-trapping fungi exposed to mycostatic factors produced traps directly from the spore without the development of mycelium. Kerry (1984) considered endoparasitic fungi producing adhesive spores or resting spores germinating only in the presence of the host to be less sensitive to mycostasis. Kerry (1984) also discussed the convenience of nutrient sources for overcoming mycostasis in applications of nematophagous fungi to control nematode populations in soil. Rhizosphere microorganisms, and specially pseudomonad bacteria have been found to inhibit the growth of several microorganisms including fungi (Schroth *et al.*, 1983; Schippers *et al.*, 1987a).

In this paper we report the growth inhibition of *V. suchlasporium*, an egg-parasite of cyst nematodes (Gams, 1988) and other nematophagous fungi by a rhizosphere bacterium, isolated from females of the cereal cyst nematode *Heterodera avenae* Woll.

Materials and methods

Heterodera avenae females and newly-formed cysts were obtained in pots by growing susceptible oats (cv. Peniarth) in naturally infested soil (Spittalfield, Blairgowrie, Scotland) known to contain endoparasitic fungi (Lopez-Llorca and Duncan, 1986).

The soil used for the pot-experiments was collected from eight points of the same field. Samples were bulked and stored at 5°C in the dark for at least 5 weeks before use to break the diapause and ensure hatching of *H. avenae* (Fisher, 1981). The soil was used directly from the cold room to fill (30 x 30 x 25 cm) square pots which were sown with oats. When the seed had germinated, seedlings were thinned so as to have approximately 20 plants per pot. Pots were then left in a greenhouse at 15°C.

Appoximately ten oat plants were removed with their roots and adhering soil from three pots at 6, 7, 10, 13, 15, 16, 17, 18 and 25 weeks after sowing. The tops of the plants were severed and the roots left for 15 min in tap water to loosen soil and debris. Afterwards, the roots systems were washed gently in tap water and checked under the microscope for the presence of white females and/or newly-formed cysts.

Nematodes were picked-off the root systems, and separated into white females and newly-formed brown cysts. White females were separated, according to their size and shape, into two age categories viz. young females (YW) (small and elongated) and older females (OW) ('lemon-shaped' and bigger than YW).

Females and cysts from the roots were washed twice in sterile distilled water (SDW) and plated on to 90 mm plastic petri dishes containing a growth-restricting medium ("Medium 1", Lopez- Llorca and Duncan, 1986) slightly modified (no rose bengal was included and penicillin was used instead of aureomycin). An average of 10 females or cysts per dish were plated. Plates were incubated at 20°C in the dark for 6-7 days. Cysts and females were then inspected for the presence or absence of fungi which were identified.

The plates with females in which fungi were developing were also scored for the presence of 'halos' of growth inhibition.

Bacterial colonies which developed from young females of *H. avenae* when plated on modified Medium 1, and those females showing mycostasis, were subcultured on nutrient agar (Oxoid) and single colonies isolated on the same medium. Bacterial colonies which differed in appearance, were sent to the Commonwealth Mycological Institute (CMI) for identification. The different bacterial colonies were also plated on corn meal agar (CMA), potato dextrose agar (PDA) and water agar (WA) (Oxoid) with egg- parasites from infected eggs of *H. avenae* and other fungi at 20°C in the dark, to test their effects on the growth of pure cultures of these fungi.

Results

The results for the fungal colonisation of *H. avenae* white females and newly-formed cysts ('brown cysts') are presented in Fig. 1. The percentage of white females colonised by fungi increased between weeks six and seven until week ten when the infection rate remained ralatively constant while the percentage of brown cysts infected by fungi was consistently above 90%.

The infection of the white females with *Verticillium* species alone gradually rose between weeks 6 and 25 while the *Verticillium* infection in the brown cysts increased from around 20% at week 15 to nearly 50% in week 17 before decreasing to about 20% at week 25.

When plated on growth-restricting medium a large proportion of young females did not develop fungus (Fig. 1a)

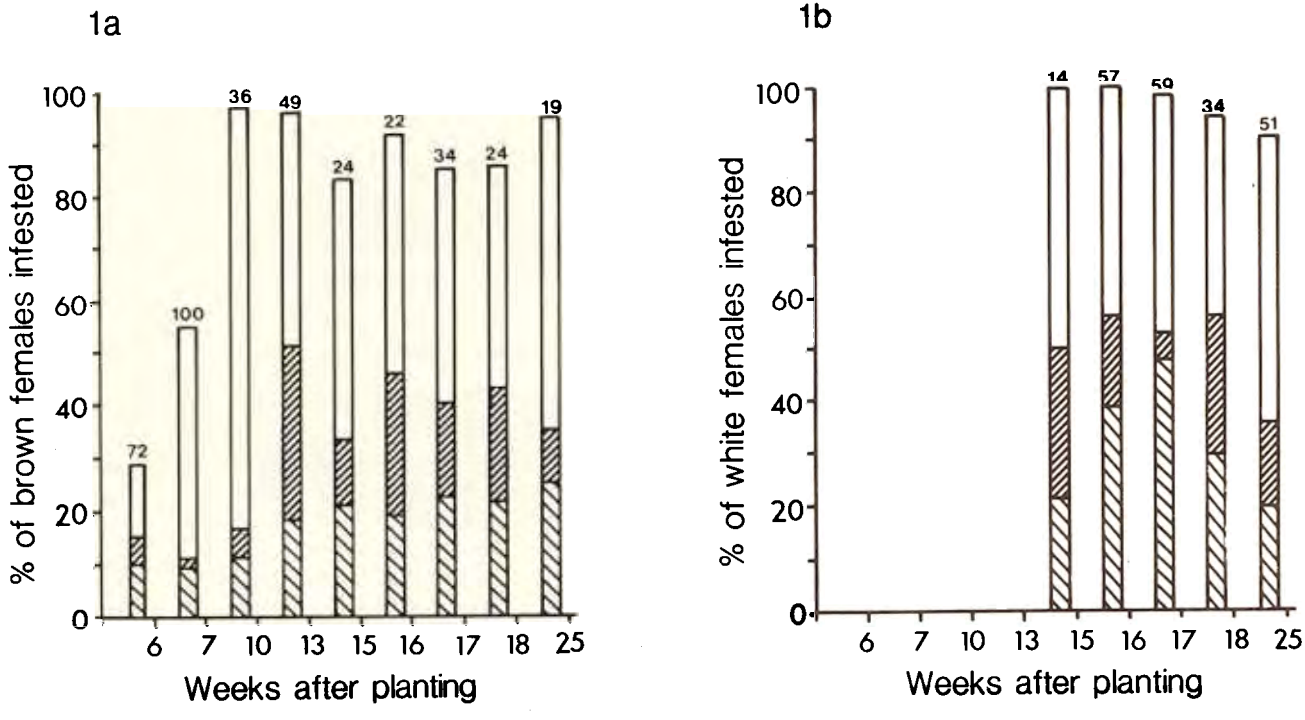

Fig. 1 - Fungal colonisation of white females (1A) and newly formed cysts (brown cysts) (1B). , *Verticillium* only; , *Verticillium* plus other fungi; , fungi other than *Verticillium*. Sample size is given above each column.

and some of these apparently had no microorganism associated with them. In others, small, shiny globules developed which suggested that they were bacterial colonies, although the media contained antibiotics. Where nematodes with and without fungi were present on the same plate growth inhibition 'halos' were associated with nematodes that had bacteria-like colonies, irrespective of whether fungal colonies had developed. In one instance a female developing *Verticillium*, itself contained a shiny colony with antifungal activity against the fungus.

Mycostasis 'phenomena' were most frequently observed in young females, six halo area being recorded from six agar plates while only one halo was observed from five plates containing cysts (Table I).

Colonies from three females of *H. avenae* showing antifungal action against females developing *V. suchlasporium* or *Acremonium* sp. were subcultured and single colonies isolated. Three colony types were observed. Isolate B was sent to the Commonwealth Mycological Institute (CMI) and was identified as *Pseudomonas fluorescens* (Trevisan) Migula, biovar 1 or A (Dr J.F. Bradbury, CMI, personal communication). Colonies of isolate A had similar characteristics to isolate B (*P. fluorescens* and probably were also a pseudomonad bacterium, but isolate A colonies were not identified and are referred to as 'isolate A'.

The bacterium 'isolate A' and *P. fluorescens* inhibited the growth of the egg-parasites *Paecilomyces carneus* and *Acremonium* sp. but not that of the phytopathogen *Cladosporium cucumerinum*. Isolate A also inhibited the growth of *V. suchlasporium* (Table II).

Figures 2a and 2b shows the growts inhibition of *Acremonium cerealis* (from *H. avenae* females) by the bacterium *P. fluorescens* on CMA and WA. Figures 2c and 2d show the same features on the same media for *V. suchlasporium* and the bacterium 'isolate A'. The plates were incubated at 20°C in the dark and photographed 20 days after inoculation.

TABLE I - *Occurrence of mycostasis in fungal colonies associated with young and old* Heterodera avenae *females.*

Plate number	Total number of females	Young females		Plate number	Total number of females	Old females	
		No. infected with fungi	No. infected with fungi and mycostasis			No. infected with fungi	No. infected with fungi and mycostasis
1	8	1	0	7	7	2	0
2	7	0	0	8	6	1	0
3	7	2	2	9	6	4	0
4	6	1	1	10	8	4	0
5	7	1	1	11	6	1	1
6	6	3	2				

TABLE II - *Antifungal activity of the two bacterial isolates* Pseudomonas fluorescens *(PF) and isolate A (A) from* H. avenae *females on the growth of* Paecilomyces carneus, Verticillium suchlasporium, Acremonium *sp. and* Cladosporium cucumerinum *on different agar mediums.*

Fungus	Agar medium		
	Water agar	Corn meal agar	Potato dextrose agar
Paecilomyces carneus	PF + *	PF +	NT
Verticillium suchlasporium	A +	A +	NT
Acremonium sp.	PF +	PF +	NT
Cladosporium cucumerinum	PF – A –	NT	A + / –

* + , growth inhibition observed; – , no growth inhibition observed; + / – , little growth inhibition observed; NT, not tested.

Discussion

Mycostasis by bacteria has also been observed in other invertebrate pathogens such as entomopathogenic fungi. Dillon and Charnley (1986) reported the inhibition of the entomophagous fungus *Metarhizium anisopilae* by insect gut bacterial flora.

The results of the present investigation indicate that *P. fluorescens*, and another bacterium, could colonise females of *H. avenae* and inhibit the growth of fungal egg-parasites. This is consistent with the observations of several other workers who also found that rhizosphere bacteria can act as natural antagonists of fungi (Schroth *et al.*, 1983; Lambert *et al.*, 1987; Schippers *et al.*, 1987a, 1987b). Ganesan and Gnanamanickam (1987) inoculated plants with *P. fluorescens* and provided protection against the phytopathogenic fungus *Sclerotium rolfsii*.

The mechanism by which fluorescent pseudomonads inhibit fungi in the soil seems to be by competition for iron. The bacteria release siderophores; these are secondary metabolites with a strong affinity for iron (Fe + 3) (Schippers *et al.*, 1987a, 1987b).

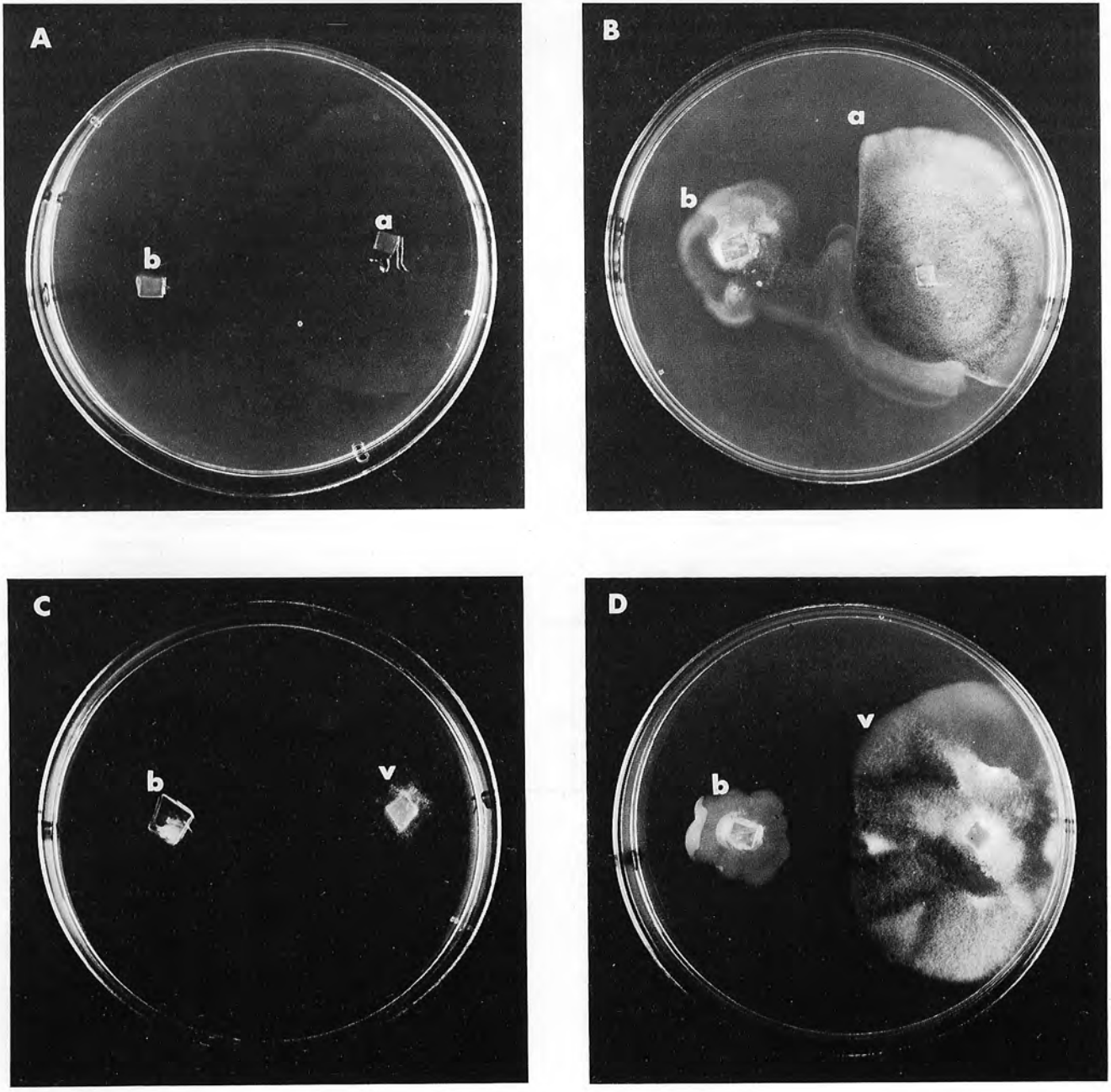

Fig. 2A-2D - Antifungal effect of rhizosphere bacteria on egg-parasites of *H. avenae*, 20 days after inoculation at 20°C in the dark: A, B, growth inhibition of *Acremonium cerealis* (a) by *Pseudomonas fluorescens* (b); (A in 1% water agar A), (B in corn meal agar). C, D, growth inhibition of *V. suchlasporium* (v) by bacterium 'isolate b' (C in 1% water agar, D, in corn meal agar).

The growth of *C. cucumerinum*, a fungal plant pathogen and not involved in cyst-nematode parasitism, was unaffected by the bacteria which inhibited growth of *V. suchlasporium* and other egg-parasites. This might indicate a certain degree of specificity in the mycostasis induced by the bacteria tested. The growth of the egg-parasitic fungi was never completely inhibited by bacteria. *V. chlamydosporium* has been shown to have antibacterial activity against several species (Filipello-Marchisio, 1976) which may explain why the antagonistic bacterium (*P. fluorescens*) was found together with egg-parasitic fungi in some *H. avenae* females.

Peterson and Katzenelson (1964, 1965) also observed differences in the degree of colonisation of wheat and soybean rhizosphere by the nematode trapping-fungus *A. oligospora*. The poorer colonisation of wheat rhizosphere by the fungus was associated with the presence of bacterial flora antagonistic to the fungus, absent in soybean rhizospere.

Differeces recorded in the root- colonisation of several species of cereals by the egg-parasites in Scotland (Lopez-Llorca, 1988) may also be due to rhizosphere mycostatic bacteria.

The percentage of nematodes colonised by nematophagous species of *Verticillium* increases from females to newly-formed cysts, supporting the view that these fungi have a major role in egg- parasitism. The increase in the occurrence of *Verticillium* with time may also indicate that the fungus overcomes mycostasis and perhaps competes with the 'other fungi', probably by producing inhibitory substances (Leinhos and Buchenauer, 1986), and thus becoming the dominant egg-parasite.

The mycostatic action of rhizosphere bacteria was mainly restricted in this study to young females (6 weeks after sowing). Mycostasis may act to protect young females from fungal parasitism, but its effects appeared to decrease progressively. However, the results shown here indicate that mycostasis is a process operating in soils which may be relevant to parasitism of cyst nematodes by egg-parasites such as *V. suchlasporium* and others.

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Literature cited

Dillon R.J. and Charnley A.K., 1986 - Inhibition of *Metarhizium anisopilae* by the gut bacteria flora of the Desert Locust, *Schistocerca gregaria*: evidence for an antifungal toxin. *J. Invert. Pathol.*, *47*: 350-360.

Filipello-Marchisio V., 1976 - Sull'attività antibiotica di *Diheterospora chlamydosporia* e di *Oidiodendron truncatum*. *Allionia*, *21*: 67-71.

Fisher J.M., 1981 - Further observations on the effect of temperature on *Heterodera avenae* Woll.. *Nematologica*, *27*: 228-234.

Gams W., 1988 - A contribution to the knowledge of nematophagous species of *Verticillium*. *Neth. J. Pl. Path.*, *94*: 123-148.

Ganesan P. and Gnanamanickam S.S., 1987 - Biological control of *Sclerotium rolfsii* Sacc. in peanut by inoculation with *Pseudomonas fluorescens*. *Soil Biol. Biochem.*, *19*: 35-38.

Jackson R.M., 1957 - Fungistasis as a factor in the rhizosphere phenomenon. *Nature (London)*, *180*: 96-97.

Kerry B.R., 1981 - Fungal parasites: A weapon against cyst nematodes. *Plant Disease*, *65*: 390-393.

Kerry B.R., 1984 - Nematophagous fungi and the regulation of nematode populations in soil. *Helminthological Abstracts, Series B, Plant Nematology*, *53*: 1-14.

Kerry B.R., 1986 - An assessment of the role of parasites and predators in the regulation of cyst nematode populations, pp. 433-450, in: Cyst Nematodes (Eds. F. Lamberti and C.E. Taylor) NATO ASI Series Life Sciences Vol. 121, Plenum Press, N. Y. and London.

Kerry B.R., Simon A. and Rovira A.D., 1984 - Observations on the introduction of *Verticillium chlamydosporium* and other parasitic fungi into soil for control of the cereal cyst nematode *Heterodera avenae*. *Ann. appl. Biol.*, *105*: 509-516.

Ko W. and Lockwood J.L., 1970 - Mechanism of lysis of fungal mycelia in soil. *Phytopathology*, *60*: 148-154.

Lambert B., Leyns F., Van Rooyen L., Gossele F., Papon Y. and Swings J., 1987 - Rhizobacteria of Maize and Their Antifungal Activities. *Appl. Env. Microbiol.*, *53*: 1866- 1871.

Leinhos G. and Buchenauer H., 1986 - Untersuchungen zur antagonistischen Wirkung des Bodenplizes *Verticillium chlamydosporium* Goddard gegenuber Getreiderostpilzen. Mitteillungen aus der Biologischen Bundesanstalt fur Land und Forstwirtschaft, p. 275. 45 Beutsche.

Lopez-Llorca L.V., 1988 - Fungal Endoparasites of Cyst Nematodes. Modes of Action. PhD Thesis. University of Dundee, pp. 263.

Lopez-Llorca L.V. and Duncan J.M., 1986 - New media for the estimation of fungal infection in eggs of the cereal cyst nematode, *Heterodera avenae* Woll.. *Nematologica*, *32*: 486-490.

Mankau R., 1962 - Nematophagous fungi and soils fungistasis. *Phytopathology*, *52*: 611-615.

Peterson E.A. and Katznelson H., 1964 - Occurrence of nematode-trapping fungi in the rhizosphere. *Nature*, *204*: 1111-1112.

Peterson E.A. and Katznelson H., 1965 - Studies on the relationships between nematodes and other soil microorganisms. IV. Incidence of nematode-trapping fungi in the vicinity of plant roots. *Can. J. Microbiol.*, *111*: 491-495.

Schroth M.N., Loper J.E. and Hildeband D.C., 1983 - Bacteria as biocontrol agents of plant disease, pp. 362-369, in: Current Perspectives in Microbial Ecology (Proceedings of the 3d International Symposium) (Eds M.J. Klung and C.A Reddy) Publ. The American Society of Microbiology.

Schippers B., Bakker A.W. and Bakker P.A.H.M., 1987a - Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Ann. Rev. Phytopathol.*, *25*: 339-358.

Schippers B., Lugtenberg B. and Weisbeek P.J., 1987b - Plant growth control by fluorescent pseudomonads, pp. 19-39, in: Innovative Approaches to Plant Disease Control (I. Chet ed.) Chapter 2, Wiley Series in Ecological and Applied Microbiology. John Wiley and Sons, N. Y.

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